Mechanism of Pressure-Induced Gelation of Milk

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The pressure-induced gelation of concentrated skimmed milk and milk−sugar mixtures was studied to discover the main components responsible for gelation. The major protein component responsible for gelation is micellar casein. Gelation occurs at similar pressures to casein micelle disintegration in dilute milk, and both can be prevented by inclusion of excess calcium chloride. Transmission electron micrographs show that the protein network is formed from particles with diameters approximately an order of magnitude smaller than those of intact casein micelles. Gelation occurs on decompression and is found to be baroreversible. Concentrations of sugar up to 30% reduce the critical concentration of casein required for gelation, but higher sugar concentrations inhibit gelation. A mechanism of gelation based on the aggregation of casein submicelles formed by pressure-induced disintegration of casein micelles is proposed. Observations on the effect of sucrose on gelation are discussed in terms of the influence of sugars on the solvent quality in aqueous casein systems.

Keywords: *Milk; casein; sugar; protein gelation; high pressure; calcium phosphate*

INTRODUCTION

The application of high-pressure processing to food systems for purposes other than preservation has been discussed by many authors (see, for example, refs *1* and *2*). Several commercial products that rely on high-pressure processing for the modification of the functional properties of one or more ingredients are widely available in Japan (*3*), and similar products are currently becoming available in Europe. To fully exploit the potential of high-pressure processing, it is important to understand the effect of pressure on the physical and chemical properties of individual ingredients. This paper reports a study into the mechanism of gelation of skimmed milk induced by high-pressure processing.

The application of high pressure to bovine milk has been studied for almost a century (*4*). Whereas early studies concentrated on the potential of high pressure for milk preservation, recent studies have shown that pressurization leads to irreversible changes in some of the physical properties of milk. For example, skimmed milk shows an irreversible increase in viscosity and decrease in turbidity after treatment at pressures >200 MPa (*5, 6*). Gelation of nonconcentrated skimmed milk (at natural pH), however, has not been observed, even when relatively severe treatments (as high as 1 GPa) have been employed (*5*).

Kumeno and co-workers were the first to show that freeze-concentrated milk, which has a solids content >25% (w/w), can be gelled by the application of pressures >200 MPa (*7*). In a mechanistic study the same group found that gelation was related to the disintegration of casein micelles at high pressure (*8*). Subsequent studies by other groups have taken a more phenomenological approach and have concentrated on the effect of various physicochemical parameters (concentration, pH,

ionic strength, and temperature) on the viscoelasticity and "strength" of the pressure-induced gels (*9, 10*). None of the studies conducted thus far have resulted in the development of a hypothesis for the mechanism of gelation, and even basic information such as whether gelation occurs on compression or decompression is lacking. Some authors have suggested that protein "denaturation" (*7*) or "conformational rearrangement" (*9*) is involved in the pressure-induced gelation of milk.

The aim of the work described in this paper was to further investigate the phenomenon of pressure-induced gelation of milk in order to construct a tangible hypothesis for the mechanism. Therefore, the experiments have been designed with the aim of identifying the major components responsible for the pressure-induced gelation of concentrated skimmed milk. The major approach adopted has been to examine the effect of composition on the structure (as determined from turbidimetry and electron microscopy) and rheology of milk protein−sugar mixtures examined after treatment at high pressure.

EXPERIMENTAL PROCEDURES

Materials. Spray-dried skimmed milk powder (SMP) produced via a medium-heat method was obtained from Eden Vale Dairies (York, N. Yorkshire, U.K.) and had the following composition: 37.0% protein, 52.0% lactose, 0.4% fat, 7.9% total ash, 1.3% calcium, and 2.7% moisture. *Bi*PRO, a commercial whey protein concentrate (WPC) that is produced from concentrating and spray-drying sweet whey with minimum heat treatment, was obtained from Davisco Foods International (Le Sueur, MN) and had the following composition: 92.5% protein, 0.2% lactose, 0.3% fat, 2.0% total ash, 0.1% calcium, and 4.9% moisture. Sodium caseinate, produced by acid precipitation of casein from fresh skim milk followed by neutralization and drying, was supplied by DMV International (Veghel, The Netherlands) and had the following composition: 89.5% protein, 0.2% lactose, 0.8% fat, 4.5% total ash, 0.1% calcium, and 5.0% moisture. Inorganic salts were obtained from Fisher Scientific (Loughborough, Leicestershire, U.K.) with the exception of magnesium citrate, which was obtained from BDH Laboratory Supplies (Poole, Dorset, U.K.). Lactose monohy-

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drate was supplied by Sherma Chemicals (Sandy, Bedfordshire, U.K.). Sucrose was food grade sugar. *N*-Ethylmaleimide (NEM) was obtained from Fisher Scientific. Phosphocaseinate (the freeze-dried retentate of microfiltered milk) is a form of native micellar casein (*11*). The phosphocaseinate used here was a gift from the INRA of Rennes (France) to C. Schorsch of our laboratory and had the following composition: 85.7% casein protein, 5.0% noncasein protein, 0.5% lactose, and 8.3% ash. All water used was deionized (18 MΩ).

Sample Preparation. Concentrations are expressed on a percent weight by weight basis unless otherwise stated.

With the exception of systems containing phosphocaseinate, mixtures were prepared by dispersing the appropriate protein-containing powder into deionized water at room temperature with vigorous agitation by a magnetic stirrer. After 30 min, the required amounts of solid sucrose and/or lactose and/or inorganic salts were added. Systems containing milk powder, sugar, and water only were used at their natural (unadjusted) pH (pH 6.2–6.5). The pH of samples containing protein concentrates and/or additional chemicals was adjusted to 6.5 with either 2 M KOH or 2 M HCl.

Phosphocaseinate-containing mixtures were prepared as follows: Lactose-free synthetic milk ultrafiltrate (SMUF) was prepared from a dry salt blend according to the method of Jenness and Koops (*12*). To this were added the appropriate amounts of lactose and phosphocaseinate. The mixture was then heated at 60 °C for 3 h with gentle agitation in a sealed flask on a stirrer hotplate. After the mixture had cooled to room temperature, sucrose and WPC (if required) were added, the pH was adjusted to 6.5 with 2 M KOH, and the mixture was agitated for a further 30 min.

Samples were stored at 2 °C for 24 h prior to pressure treatment.

Pressure Treatment. Pressure treatment was performed isostatically at room temperature (20 °C) using either a 1 L capacity press (EPSInt) or a 200 mL capacity press (Stansted Fluid Power). For pressure treatment, samples were sealed in 30 mL low-density polythene bottles. These bottles were filled to maximum capacity to ensure minimum headspace. The pressure-transmitting fluid was water in the case of the EPSInt press and ethanol in the case of the Stansted press. The thermal inertia in both of these presses is large enough for compression and decompression to be almost adiabatic at the rates used (typically 7 MPa s^{-1} for both compression and decompression). Therefore, owing to the difference in compressibility between water and ethanol, the associated temperature change of the pressure-transmitting fluid owing to adiabatic volume change was around 0.02 and 0.03 K MPa^{-1}, respectively. Despite this difference, the change in sample temperature, monitored using a specially designed thermocouple (*13*), was ~0.02 K MPa^{-1} irrespective of the fluid used. In addition, thermal equilibrium was reached within 10 min in both presses such that the sample and fluid temperatures were within 1 °C of the ambient temperature. Therefore, the pressure–temperature histories of the samples were effectively identical regardless of the press or fluid used.

Rheometry. All rheological measurements were performed on a Carrimed CSL500 rheometer operating with a cone-and-plate geometry. A 6 cm diameter acrylic cone with an angle of 2° and truncation of 53 μm was used throughout.

The oscillatory rheology of samples was measured at 5 °C (in order to minimize sample drying caused by evaporation of water) ~2 days after pressure treatment by performing a log frequency sweep at a constant strain of 0.005. Some samples that were of very low viscosity failed to give a response greater than the instrument noise at 0.005 strain. The rheology of these low-viscosity samples was characterized at a constant stress of 5 Pa (after it was first confirmed that this stress was in the linear viscoelastic regime by performing a stress sweep from 0.1 to 10 Pa). All rheological parameters are quoted at a frequency of 1 Hz. Samples were considered to be gelled if the measured storage modulus, G', was greater than the loss modulus, G'' (i.e., if tan δ was <1) at this frequency. The dynamic viscosity, η' (= G''/ω, where ω is angular frequency), was also used to characterize the samples. For selected

samples, the oscillatory rheology was measured (at 20 °C) as a function of time after decompression by performing a time sweep. The stress was set at 0.1 Pa and frequency at 1 Hz. Sunflower oil was used to cover the exposed edges of the sample during the time sweep to eliminate evaporation of water.

The degree of reproducibility and estimated error in each rheological parameter varied according to whether the system was a gel or a low-viscosity liquid. For gels and liquids viscous enough to allow measurement at a strain of 0.005, a major source of experimental error arises from damage to the sample during loading onto the rheometer. This results in a large variance in the measured values of G', G'', and η'. For these parameters, the relative standard deviation (RSD) for three measurements on a given system was of the order of 50%. Values of the loss tangent, however, were much more reproducible (RSD ~ 10%) as reduction in G' owing to sample loading effects was accompanied by a corresponding reduction in G''. Low-viscosity systems (i.e., those that did not give a measurable response at a stress of 0.005) are less sensitive to sample loading effects, and in these cases the major error arises from the fact that the elastic moduli are of the order of the instrument noise. Therefore, in the case of low-viscosity liquids, an RSD as large as 50% was typical for G', whereas for G'' and η' the RSD was ~10% (owing to the more favorable signal-to-noise ratio for these parameters). As a consequence of a large variance in G' with a relatively low variance in G'', the resulting error in the loss tangent was relatively high for very liquid systems (~50%).

Native Whey Protein Determination. In this work, native whey protein is defined as that soluble at pH 4.6. The amount of native whey protein in a given sample was determined as follows: 30 mL of sample was weighed into a 50 mL glass beaker. With stirring, the pH was adjusted to 4.6 by the addition of 1 M HCl and the sample made up to 50 mL with deionized water. Forty grams of this sample was then centrifuged at 3500 rpm for 20 min. The supernatant was then removed and filtered through Whatman No. 541 filter paper followed by a 0.2 μm membrane filter prior to separation and quantification of the whey proteins by capillary electrophoresis (CE).

CE was conducted using a Hewlett-Packard HP^{3D} capillary electrophoresis system operating with a 50 μm internal diameter capillary (50 cm effective length) coated with a neutral hydrophilic phase (Supelco CElect P150). Samples were injected hydrodynamically at a pressure of 50 mbar for 5 s and electrophoresed at 25 kV for 15 min at a temperature of 45 °C. The run buffer used was 50 mM sodium phosphate, pH 2.5, and detection was at 214 nm. New capillaries were equilibrated with 1 M sodium hydroxide for 30 min, 0.1 M sodium hydroxide for 30 min, and running buffer for 3 h. All capillaries were rinsed for 2 min with water and for 2 min with run buffer between runs. Quantification was performed by analysis of peak areas relative to standards containing purified α-lactalbumin and β-lactoglobulin (A and B) from Sigma. The typical RSD of the peak areas for three repeat injections was 4%, leading to an uncertainty of ~8% in the calculated concentration values.

Optical Density Measurements. The optical densities of two systems (14.4% SMP and 14.4% SMP/17.6% sucrose) treated at 400 MPa for 40 min were followed as a function of time after the release of pressure. Measurements were made on undiluted samples at 600 nm in a 2 mm path length cell using a CECIL CE 292 spectrophotometer. The complete experiment (i.e., starting with sample preparation) was performed on two separate occasions, and for each system the reproducibility of the optical density at a given time after pressure release was better than 0.1 absorbance unit.

Hydrophobicity Determination. Estimation of the surface hydrophobicity of milk proteins was carried out according to the procedure of Bonomi et al. (*14*) using the ammonium salt of the fluorescent dye 8-analino-1-naphthalenesulfonic acid (ANS) supplied by Fluka (Poole, Dorset, U.K.). Briefly, the sample was diluted 1/10 with potassium phosphate buffer (pH 6.8) and dispersed thoroughly with stirring on a magnetic

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Table 1. Effect of Treatment at 400 MPa for 40 min on the Physical State and Rheology of Several Milk Protein-Containing Systems[a]

system	state	tan δ	G' (Pa)	η' (Pa·s)
14.4% SMP	sol	70 ± 40	$(1.6 ± 0.9) \times 10^{-4}$	0.014 ± 0.002
14.4% SMP/17.6% sucrose	gel	0.21 ± 0.03	$(4 ± 2) \times 10^{1}$	1.4 ± 0.7
14.4% SMP/17.6% sucrose/50 mM EDTA	sol	60 ± 20	$(3 ± 1) \times 10^{-3}$	0.026 ± 0.003
4.5% sodium caseinate/1% WPC/7.5% lactose/17.6% sucrose	sol	170 ± 70	$(8 ± 3) \times 10^{-4}$	0.020 ± 0.002
4.5% sodium caseinate/7.5% lactose/17.6% sucrose	sol	200 ± 100	$(9 ± 5) \times 10^{-4}$	0.018 ± 0.001
4.5% phosphocaseinate/1% WPC/7.5% lactose/17.6% sucrose in SMUF	gel	0.26 ± 0.06	$(1.2 ± 0.1) \times 10^{2}$	4.7 ± 0.6
4.5% phosphocaseinate/7.5% lactose/17.6% sucrose in SMUF	gel	0.22 ± 0.01	$(1.4 ± 0.5) \times 10^{2}$	5 ± 2
1% WPC/7.5% lactose/17.6% sucrose	sol	12 ± 9	$(1.4 ± 0.9) \times 10^{-2}$	0.04 ± 0.04
4.5% WPC/1% sodium caseinate/7.5% lactose/17.6% sucrose	sol	14 ± 2	$(8 ± 1) \times 10^{-3}$	0.017 ± 0.001
14.4% SMP/17.6% sucrose/13 mM NEM	gel	0.17 ± 0.01	$(1.7 ± 0.2) \times 10^{2}$	4.6 ± 0.7
14.4% SMP/0.5 M K$_2$SO$_4$	gel	0.35 ± 0.04	$(7 ± 3) \times 10^{1}$	4 ± 2
14.4% SMP/0.5 M KCl	sol	14 ± 3	$(8 ± 2) \times 10^{-3}$	0.019 ± 0.002
14.4% SMP/1.5 M KCl	gel	0.20 ± 0.01	$(4.4 ± 0.7) \times 10^{2}$	14 ± 2

[a] Measurements were made at constant stress (5 Pa) for sols and at a constant strain (0.005) for gels. Values given are the mean of three measurements with the associated 95% confidence interval.

stirrer. The sample was then titrated with a solution of ANS until the fluorescence intensity of the protein−ANS complex (excitation, 390 nm; emission, 480 nm) reached a maximum. This maximum intensity, F_{max}, was taken as a measure of the number of exposed hydrophobic binding sites per unit volume.

Reproducibility of the experiment was checked using a 14.4% SMP/17.6% sucrose system treated at either 100, 300, or 400 MPa for 40 min. For two samples of this system, prepared and pressure-treated separately, the reproducibility of F_{max} for a given pressure treatment was better than 200 fluorescence units.

Transmission Electron Microscopy. A sample comprising an aqueous dispersion of 14.4% SMP and 17.6% sucrose was prepared and treated at 400 MPa for 40 min. An untreated sample of the same mixture was used as control. This control and the pressure-induced gel were stored at 2 °C for 4 days prior to chemical fixing.

Owing to its low viscosity, the control sample was pre-embedded in 2% agar prior to fixing. Samples were fixed by placing small cubes (∼4 mm³) in 1.0% glutaraldehyde/0.05% formaldehyde in phosphate-buffered saline, pH 7.5 (Sigma), for 4 h at 4 °C. The samples were then washed twice in phosphate-buffered saline and left overnight in the buffer at 4 °C. Samples were then washed twice with water prior to dehydration through an ethanol series (50, 70, and 90% for 15 min each and 100% alcohol for 30 min at 4 °C). Following dehydration, embedding was performed using an LR Gold/GMA resin at 4 °C, changing for fresh resin daily for 4 days. LR Gold (London Resin Co.) was mixed with GMA (HEMA low acid, TAAB Labs) in a 6:4 ratio (per volume) together with 0.1% benzoin ethyl ether initiator (Polysciences). The resin was polymerized in gelatin capsules by UV irradiation (360 nm) for 18 h at 20 °C in a nitrogen atmosphere. Ultrathin sections (∼60 nm) were mounted onto colloidion-coated (2% colloidion in amyl acetate) nickel grids and counterstained with 2% aqueous uranyl acetate followed by Reyndd's lead citrate. Images were obtained on a JEOL 1220 transmission electron microscope with an accelerating voltage of 80 keV.

RESULTS AND DISCUSSION

Role of Casein. A model system with an arbitrary composition (14.4% SMP and 17.6% sucrose) within the range found to be gelled by pressure treatment was used to investigate the mechanism of gelation. The range of protein and sugar concentrations studied is described in the section dealing with the role of sugar.

The link between casein micelles and gelation of aqueous milk/sucrose systems is illustrated by the first nine entries in Table 1. The systems described in this table are based on the model system of 14.4% SMP/17.6% sucrose. Taking the composition of the SMP and assuming that 80% of the milk protein is casein (*15*), this model consists of approximately 7.5% lactose, 4.5%
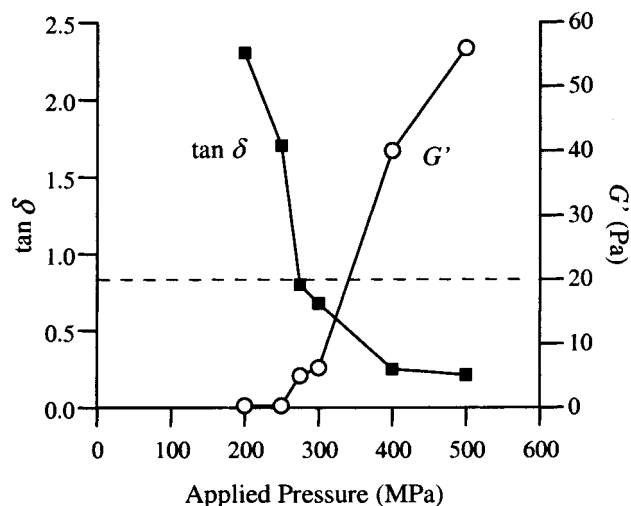
Figure 1. Loss tangent and storage modulus of 14.4% SMP/17.6% sucrose gels formed by treatment for 40 min at the pressures shown. Systems with tan δ < 1 (broken line) are considered to be gelled. Measurements were made at a constant strain of 0.005.

casein, 1% whey protein, and 17.6% sucrose. Of the systems described by the first nine entries in Table 1, only those containing intact micellar casein (in the presence of 7.5% lactose and 17.6% sucrose) form gels when treated at 400 MPa for 40 min. The substitution of micellar casein with casein showing a lower degree of association, that is, sodium caseinate or EDTA-treated casein, results in systems which show very little rheological change on pressure treatment. Similarly, it has been reported previously (*8*) that pressure-induced gelation of freeze-concentrated milk can be inhibited by disruption of micellar structure using EDTA or urea. Therefore, it appears that, in order for gelation to occur, the conditions at atmospheric pressure must be such that the caseins form supramolecular assemblies (e.g., micelles) with both themselves and colloidal calcium phosphate (CCP). The next stage is to establish the effect of pressure on these structures and the interactions that form them.

The variation of the loss tangent with the pressure applied to form gels of 14.4% SMP/17.6% sucrose systems is shown in Figure 1. Significant changes to the rheology occur only above pressures of 200 MPa, which is interesting as it is well-known that the application of pressures >200 MPa to nonconcentrated milk results in the irreversible disintegration of casein

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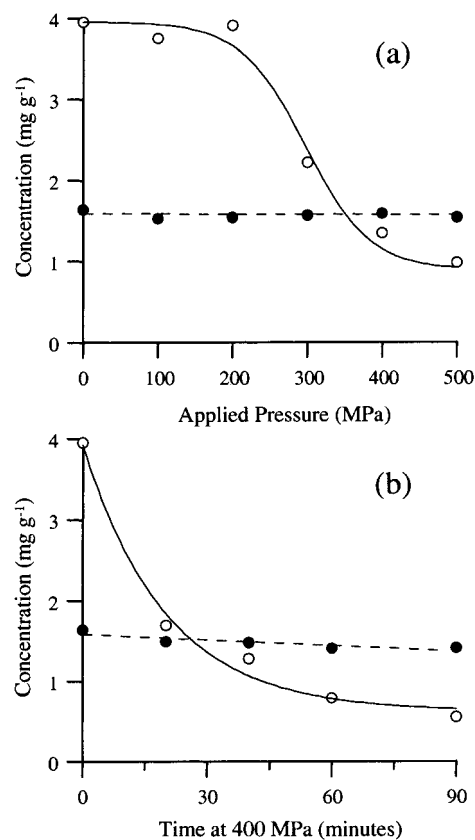*J. Agric. Food Chem.,* Vol. 49, No. 7, 2001 **3397**
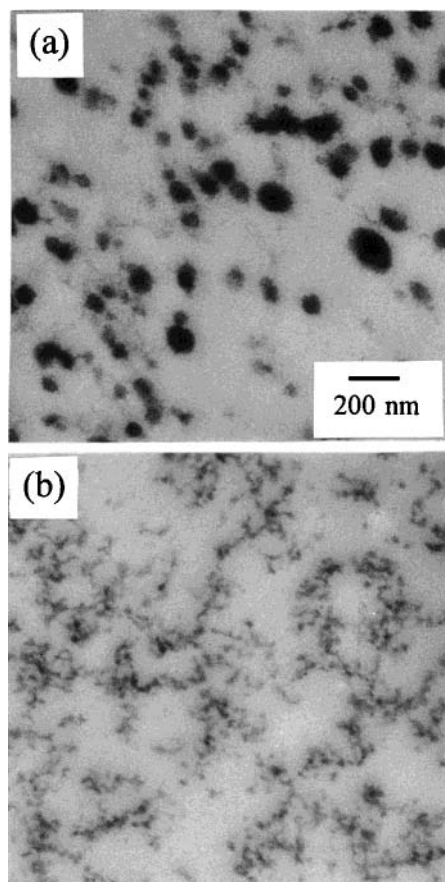
Figure 2. Transmission electron micrographs of 14.4% SMP/17.6% sucrose before (a) and after (b) treatment at 400 MPa for 40 min. Both micrographs are shown at the same magnification.

Figure 3. Effect of (a) various pressures at constant treatment time (40 min) and (b) various treatment times at constant pressure (400 MPa) on the amount of whey protein soluble at pH 4.6 in 14.4% SMP/17.6% sucrose dispersions: (●) α-lactalbumin; (○) β-lactoglobulin.

micelles (*5, 6, 16, 17*). The average diameter (as measured by light scattering) of the particles produced by pressure treatment of skim milk becomes progressively smaller with increasing pressure up to ∼500 MPa (*6*). The rheological behavior of concentrated mixtures of milk and sucrose changes over the same pressure range with a transition from viscous to viscoelastic behavior occurring when treated at pressures >200 MPa. As shown in Figure 1, this behavior becomes more elastic with increasing pressure up to 500 MPa, with a sol–gel transition occurring in the region of 250–275 MPa. These results lead to the hypothesis that the pressure-induced gelation of milk–sugar mixtures may well be related to micelle disintegration under high pressure and subsequent reassociation on pressure release.

Examination of the protein network of a pressure-induced SMP/sucrose gel by transmission electron microscopy provides further evidence for micelle disintegration and reaggregation of the resulting fragments being at the heart of the mechanism of gelation. Figure 2 shows that the network is composed of particles with diameters approximately an order of magnitude smaller than those of the intact casein micelles.

At atmospheric pressure, casein micelles are disintegrated by the lowering of calcium activity, for example, by the use of Ca^{2+}-sequestrating agents or dialysis against a Ca^{2+}-free buffer, and the small aggregates produced are often referred to as submicelles (*18*). Processes, such as the dissolution of CCP, that lead to the formation of ions in aqueous solution are generally found to have large negative reaction volumes and so

are favored by increased pressure (*19*). Therefore, it may be argued that the pressure-induced disintegration of casein micelles occurs because of a shift in the equilibrium between free and colloidal calcium and that the resulting fragments are submicelles. Consistent with this hypothesis, recent experiments by Schrader, Buchheim, and co-workers on artificial casein micelles have pointed to the importance of pressure-induced changes to the equilibrium between free and colloidal Ca^{2+} in causing micelle disintegration (*20, 21*). Entirely in agreement with these findings, we have conducted preliminary experiments on SMP/sucrose mixtures with added $CaCl_2$ and found that the presence of excess Ca^{2+} during pressure treatment prevents any significant change in either turbidity or rheology of the treated samples. Therefore, we suggest that the small particles visible in Figure 2 are casein submicelles.

Role of Whey Protein. Given that the results discussed above suggest that micellar casein is required for gelation of concentrated milk/sucrose systems, there remains the question as to whether the other proteins present in milk (the whey proteins) are also required.

The native structures of the whey proteins β-lactoglobulin (BLG) and α-lactalbumin (ALA) begin to unfold at pressures >∼100 MPa (*22, 23*). In addition, the unfolded BLG irreversibly forms disulfide-linked aggregates under pressure (*24*). Therefore, as shown in Figure 3, the amount of native BLG (as defined as that BLG soluble at pH 4.6) in SMP/sucrose systems decreases upon pressure treatment. This aggregation can be prevented by the presence of highly reactive species, which preferentially bind to any exposed thiol (−SH)

Table 2. Effect of Pressure Treatment and NEM on the Concentration of Whey Proteins in the Serum after Precipitation of Caseins at pH 4.6[a]

system	[BLG] (mg g^{-1})	[ALA] (mg g^{-1})
14.4% SMP/17.6% sucrose	4.2 ± 0.3	2.0 ± 0.2
Pressure-treated 14.4% SMP/17.6% sucrose	1.3 ± 0.1	2.0 ± 0.2
14.4% SMP/17.6% sucrose/13 mM NEM	4.2 ± 0.3	2.1 ± 0.2
Pressure-treated 14.4% SMP/17.6% sucrose/13 mM NEM	4.1 ± 0.3	2.4 ± 0.2

[a]Values given are the mean of three measurements with the associated 95% confidence interval.

groups. One such well-known thiol-blocking agent is NEM, which, when present in excess, has been reported to prevent the irreversible pressure-induced aggregation of BLG (*24*). Table 2 shows the effect of NEM on the amount of native whey protein in a pressure-treated SMP/sucrose system. As can be seen, inclusion of NEM in the system prevents pressure treatment from decreasing the amount of BLG soluble at pH 4.6. Even so, as shown by the 10th entry in Table 1, the inclusion of NEM does not prevent gelation. These results imply that whey protein aggregation (whether self-aggregation or the formation of casein–whey protein aggregates) is not a cause of pressure-induced gelation of concentrated SMP/sucrose systems. The fact that a whey protein-free micellar system does gel (seventh entry in Table 1) supports this conclusion. Further work needs to be done, however, to investigate the extent to which formation of mixed whey protein–casein aggregates affects the physical properties of the gels.

Role of Sugar. It is well established that pressure treatment of skimmed milk at physiological concentrations produces an irreversible increase in viscosity concurrent with the disintegration of casein micelles (*5, 6*) but does not result in gelation. Increasing the concentration of milk solids in raw milk to a level of 25% or higher by freeze concentration has been shown to give systems that can be gelled by pressure treatment (*7*). In addition, it has been reported that sugars affect the properties of gels made from freeze-concentrated milk (*7, 8*). We show here that sugar also has a dramatic effect on gelation of SMP dispersions.

At constant sugar concentration, the strength of the gel formed by pressure treatment depends on the concentration of casein. The data in Figure 4 show that in a system containing 7.7% lactose and 17.6% sucrose, the critical gelling concentration of casein, c_0, is ~3% for treatment at 400 MPa for 40 min. The dependence of this critical concentration on the concentration of sugar in the solvent is illustrated by the data in Figure 5. It can be seen from Figure 5a that the SMP–sucrose sol–gel state diagram is complex. However, if the state diagram is drawn as casein concentration versus total sugar concentration (where this includes the amount of lactose added with the SMP), a simpler relationship is obtained. Figure 5b shows that, up to 30% total sugars, increasing the amount of sugar in the solvent decreases c_0. Above this sugar concentration, the trend is reversed until at >40% total sugars it was not possible to produce a gel at the casein concentrations and pressure regime studied. This behavior is reminiscent of the effect of alcohols on the stability of proteins and synthetic polymers in aqueous solution at atmospheric pressure and 25 °C. Low alcohol concentrations destabilize such solutions, whereas higher concentrations have the opposite effect (*25*).
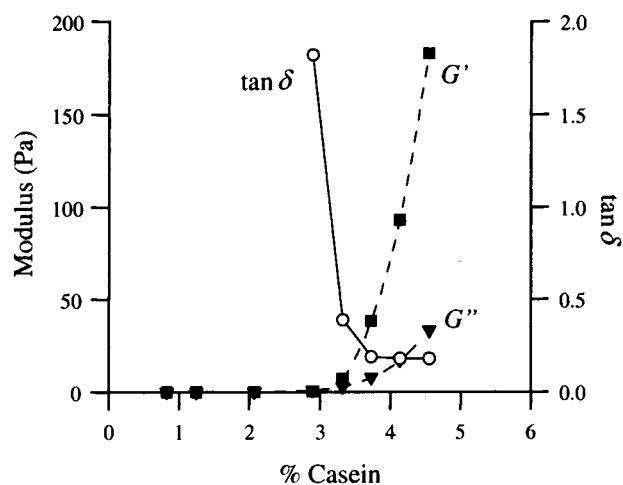

Figure 4. Viscoelastic parameters as a function of casein concentration for SMP dispersions treated at 400 MPa for 40 min with a constant concentration of total sugars (7.7% lactose/17.6% sucrose). Measurements were made at a constant strain of 0.005.
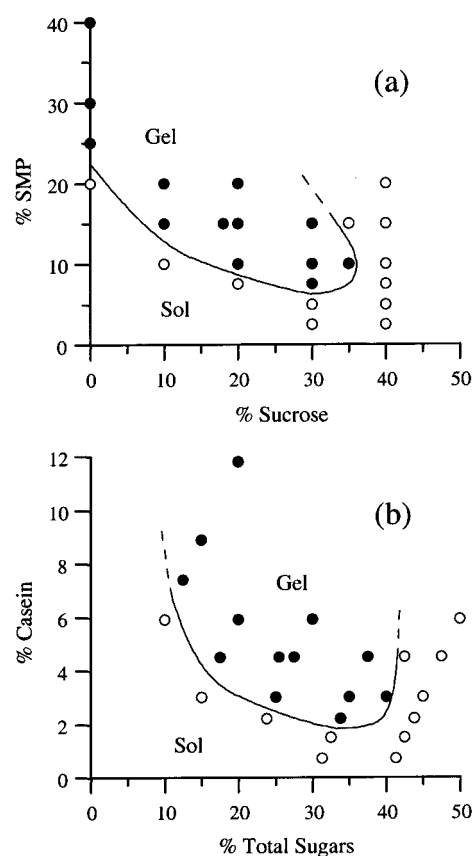

Figure 5. State diagram for systems treated at 400 MPa for 20 min. Diagram b was calculated from diagram a as described in the text. The solid circles represent compositions for which tan δ < 1.

The major differences between systems separated by the sol–gel boundary appear to evolve on pressure release. Figure 6 shows that the turbidity of both a nongelling and gelling system increases with time after pressure release (t_0 is the time at which the pressure reached 300 MPa on the decompression cycle). Electron micrographs of pressure-treated milk often show structures intermediate between micelles and submicelles (*5, 17, 18*), which have been described as chains and clusters of submicelles (*5*). The turbidity data in Figure

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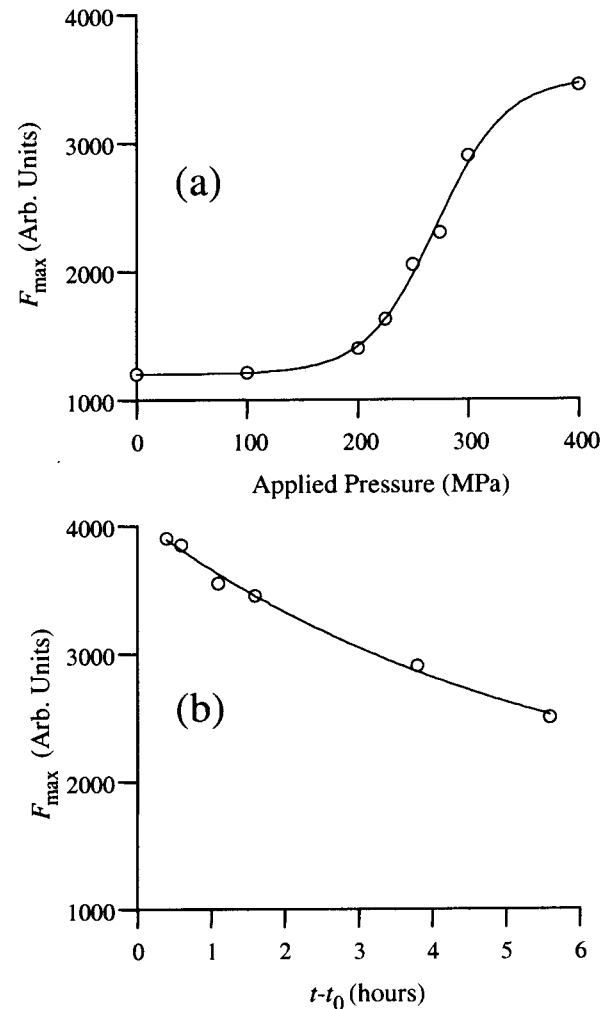*J. Agric. Food Chem.,* Vol. 49, No. 7, 2001 **3399**
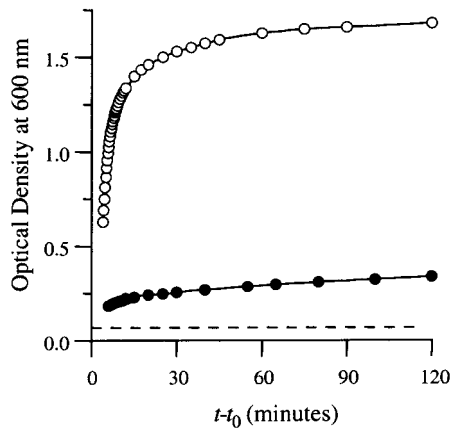
Figure 6. Evolution of turbidity with time after pressure release for 14.4% SMP (●) and 14.4% SMP/17.6% sucrose (○) treated at 400 MPa for 40 min. The dashed line shows the optical density of a sample of 14.4% SMP/50 mM EDTA at atmospheric pressure.
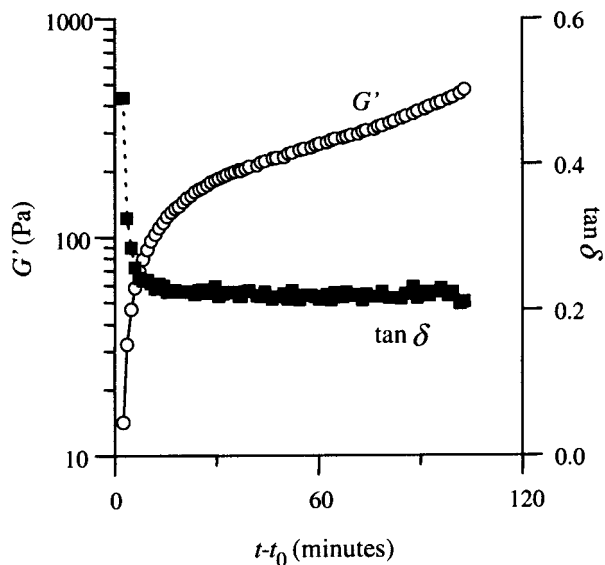
Figure 7. Evolution of rheology with time after pressure release for 14.4% SMP/17.6% sucrose treated at at 400 MPa for 40 min. Measurements were made at a constant stress of 0.1 Pa.

Figure 8. Variation of the amount of ANS–protein complex (expressed as the intensity of the fluorescence of the complex in the presence of excess ANS—see text) with (a) treatment pressure and (b) time after pressure release for 14.4% SMP/ 17.6% sucrose. Pressurization was for 40 min in all cases. For (a) titrations were performed ∼30 min after pressure release, and for (b) treatment pressure was 400 MPa.

6 for the sol sample could be extrapolated to that of a submicellar system (a system treated with 50 mM EDTA) at zero time, that is, under pressure. This implies that the increase in turbidity observed after pressure release in the case of the nongelling sol system may be caused by aggregation of free submicelles into chains and clusters of submicelles. The turbidity of the gelling system can also be extrapolated to that of a submicellar system at zero time. However, the increase in turbidity with time after decompression is much greater for this sample than that of the nongelling sample. This much larger turbidity increase results from gelation which involves the formation of spatial heterogeneities (i.e., network strands and/or pores in the network) on a length scale larger than the typical diameter of a "cluster" of submicelles present in the nongelling system.

This idea of gel formation on pressure release is supported by the rheological data shown in Figure 7. As can be seen, the storage modulus of the pressure-treated model milk/sucrose system increases and tan δ decreases rapidly after pressure release. Extrapolation

of these parameters to zero time gives values consistent with a liquid ($G' = 0$ and tan $\delta > 1$), clearly indicating that gelation occurs on decompression.

Another parameter that is found to vary with time after pressure release is the number of exposed hydrophobic sites as determined by titration with ANS. Figure 8a shows that, for the model system, the number of exposed sites increases with pressure >200 MPa, reaching a plateau above 400 MPa. This increase in hydrophobicity is probably caused by an increase in the surface area of the casein aggregates owing to disruption of the micelles. The surface of a native casein micelle is believed to be covered by a stabilizing layer of hydrophilic κ-casein "hairs". If these micelles are disintegrated into submicelles, then both the total surface area and the proportion of surface not covered by this hydrophilic coating increase, leading to the observed increase in hydrophobicity. For skimmed milk at physiological (nongelling) concentrations it has been reported that this pressure-induced increase in hydrophobicity persists for at least 8 days (*26*), but in the case of the gelling systems studied here no difference in hydrophobicity of the pressure-treated sample relative to the untreated sample could be detected after 2 days. This is because,

as shown in Figure 8b, the hydrophobicity of the pressure-treated sample decreases with time after decompression owing to aggregation of the submicelles in the gelation process.

As the sol−gel transition occurs on decompression and the properties of gelling and nongelling systems become more alike with decreasing time after decompression, it may be postulated that the role of sucrose in the pressure-induced gelation of milk is to decrease the solvent quality, at atmospheric pressure, for the form of casein (e.g., submicelles) produced by pressure treatment. The term "solvent quality" used here refers to the effect of the solvent on the net strength of the protein−protein interactions. These interactions are usually quantified using the second osmotic virial coefficient and are sensitive to both nonspecific effects (e.g., ionic strength) and specific effects. The influence of added ions and molecules on the solvent quality for proteins follows the lyotropic series (*25*). Preliminary results have been obtained for the influence of ions, occupying different places in the lyotropic series, on the pressure-induced gelation of SMP solutions. According to this series, aqueous sulfate is a much poorer solvent than aqueous chloride at an identical molal concentration. Thus, as illustrated by entries 11 and 12 in Table 1, 14.4% SMP is gelled by pressure treatment in the presence of 0.5 molal sulfate but not in the presence of 0.5 molal chloride. Entry 13 in Table 1 shows that at an ionic strength of 1.5 molal (equivalent to that of 0.5 molal K_2SO_4) 14.4% SMP treated in the presence of KCl also forms a gel. These ionic strengths are much higher than those naturally present in milk [milk ultrafiltrate has an ionic strength of ∼0.08 molal (*12*)].

The conclusion of the above discussion is that it is only on the release of high pressure that sucrose promotes aggregation and gelation of casein in aqueous SMP dispersions. It should be noted, however, that in attempts to simulate this effect, by addition of sucrose to pressure-treated SMP dispersions, gelation was never observed, even when the sucrose was added as soon as possible after decompression (6 min). This result implies that either (i) changes occur rapidly (<6 min) after decompression in the SMP system, which result in casein aggregates with a less sucrose-sensitive surface, or (ii) changes occur under pressure which are effected by the presence of sucrose in the solvent, for example, sucrose may affect the mixing behavior of the individual caseins or the solubility of calcium phosphate. The change in turbidity on decompression in sucrose-free systems (see Figure 6) seems to support hypothesis i, although clearly further investigation is required.

It is worth commenting here that the pressure-induced gelation of casein is baroreversible. As shown in Figure 9, repressurization of a pressure-induced milk/sucrose gel results in "melting" of the gel, which re-forms on subsequent decompression. The reproducibility of the gelation profile implies that the system returns to a well-defined equilibrium state under high pressure.

Proposed Mechanism of Gelation. The results described in this paper lead us to propose the scheme presented in Figure 10.

To begin with, casein and calcium phosphate are required to be associated into supramolecular structures at atmospheric pressure. In untreated milk these structures are casein micelles, but the fact that gelation is baroreversible suggests that the exact structure is
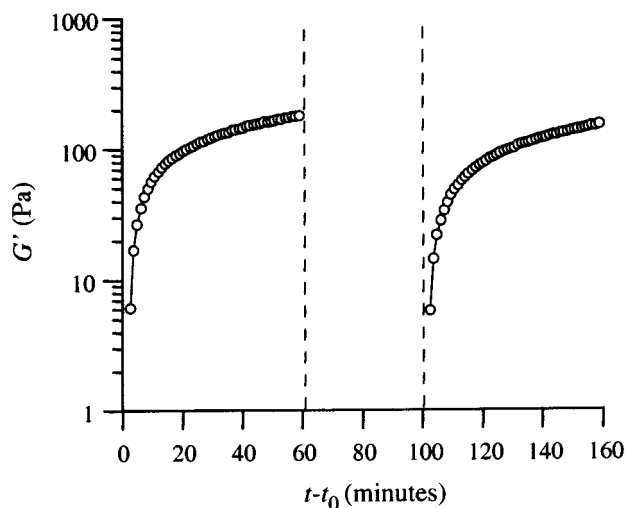
Figure 9. Evolution of storage modulus with time after decompression, demonstrating the effect of repressurization for 14.4% SMP/17.6% sucrose. Repressurization occurred for the time enclosed by the broken lines. Pressurization was at 400 MPa for 40 min in both cases. Measurements were made at a constant stress of 0.1 Pa.
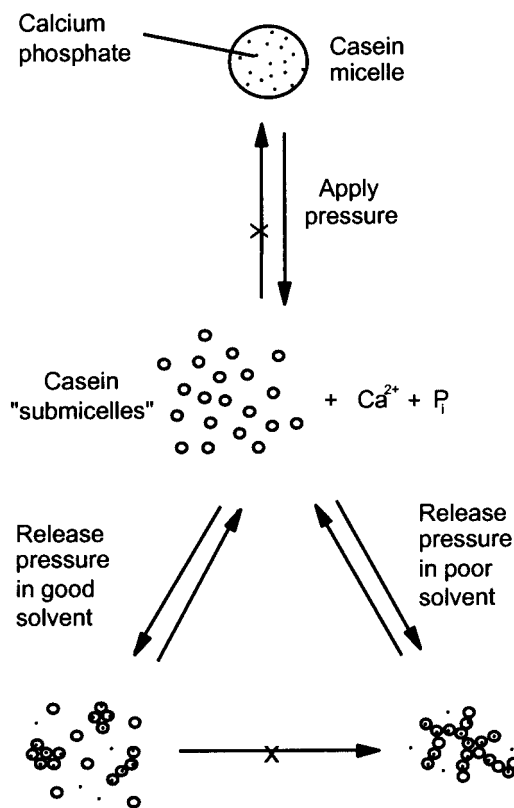
Figure 10. Schematic diagram illustrating the proposed mechanism of pressure-induced gelation of milk. (P_i = aqueous inorganic phosphate species.)

unimportant. The effect of applying hydrostatic pressure to such systems can be accounted for by assuming that the chemical potential of "free" calcium and phosphate is lowered relative to that of CCP with increased pressure. This causes dissociation of the large casein−calcium phosphate aggregates into free mineral ions and smaller casein aggregates (submicelles). It is assumed that the integrity of the submicelles is not substantially altered at high pressure. This assumption is based on the reported findings that hydrophobic association of

casein molecules is at least as strong at pressures >200 MPa as it is at atmospheric pressure (although some dissociation occurs at lower pressures) (*27–29*).

On decompression the major differences between the gelling and nongelling systems evolve. In both cases, lowering the pressure increases the activity of the free ions, causing the formation of calcium phosphate and casein–calcium phosphate complexes. This association of the submicelles with calcium phosphate increases the strength of the attractive intersubmicelle interaction. For high concentrations of casein and sugars, this interaction is strong enough for the system to be kinetically trapped into a percolated network—a gel. The origin of the larger strength of the intersubmicelle attraction in systems containing moderate amounts of sugar could be either a higher calcium activity in the aqueous sucrose solvent, leading to increased reassociation of casein and calcium phosphate, or a lowering of the solvent quality for the submicelle–calcium phosphate aggregates.

The final facet of the scheme displayed in Figure 10 to be discussed is the inability to produce the gel state from a pressure-treated sucrose-free system to which sucrose is added post-decompression. We suggest that the underlying cause may be traced to a weak interparticle attraction immediately on decompression owing to good solvent conditions in the sucrose-free system. The good solvent conditions would mean that the average lifetime of a submicelle–submicelle bond would be short. Therefore, clusters of submicelles rapidly become compact and can also "rearrange" to leave the less hydrophobic particles (e.g., submicelles with a higher content of κ-casein) at the surface, as in "native" micelles. Therefore, when sucrose is subsequently added to the system, the clusters are protected against aggregation.

Although the mechanism described above accounts for all of the experimental observations reported in this paper, it should be noted that these observations have been made following pressure treatment. To our knowledge, there have been no studies into the influence of sucrose on the quality of aqueous solvents *under* high pressure, and it may be that there is some important effect that we have not accounted for. Clearly, an investigation into the effect of sucrose on the hydration, aggregational state, and stability of caseins at both atmospheric and elevated pressure will resolve this matter.

Finally, it is worth speculating on the implications of the proposed mechanism for practical applications. Conventionally, casein is aggregated using cheese and yogurt technology to produce a variety of gels with widely different structures and rheologies. If the mechanism presented here is correct, then it may be that pressure-induced gelation produces additional structures inaccessible via conventional methods of gelling caseins. The reason for this prediction is that, in the suggested mechanism, upon decompression we have a random dispersion of submicelles that (almost) instantaneously becomes unstable. This is in contrast to conventional methods of gelation, for example, via rennetting or acidification, where already-formed clusters of submicelles (e.g., intact micelles) are aggregated slowly by a gradual increase in the interaction strength. It may be, therefore, that pressure-induced casein gels have unique properties such as texture and response

to processing. Investigations into the rheology of such systems are currently underway in our laboratory.

Conclusions. The pressure-induced gelation of concentrated skimmed milk and milk–sugar dispersions has been studied by investigating the effect of composition on the structure and rheology of the gels. Only systems containing casein and CCP (i.e., SMP or phosphocaseinate) are found to give rise to pressure-induced gels at the concentrations studied. Dissolution of CCP in SMP dispersions using EDTA or replacement of SMP with casein that does not contain CCP (sodium caseinate dispersions) produces systems that do not give rise to gels. Prevention of whey protein aggregation in SMP dispersions using a thiol-blocking agent (NEM) does not inhibit gelation. Therefore, casein micelles are the protein component required for gelation.

Gelation of concentrated SMP/sucrose mixtures occurs at pressure coincident with those found to give rise to the disintegration of casein micelles in dilute milk. Transmission electron micrographs show that the protein network is composed of particles much smaller than intact casein micelles. The turbidity and elasticity of gelling systems are both found to increase with time following the release of pressure. Similarly, the amount of protein–ANS complex decreases with time after pressure release. These results suggest that the major effect of high-pressure processing is to dissociate casein micelles into fragments that aggregate on the release of pressure.

Concentrations of sucrose to 30% are found to lower the critical concentration of casein required for gelation. Both potassium sulfate and potassium chloride at sufficiently high ionic strength also promote gelation. Explanations of these cosolute effects based on the influence of solutes on the solvent quality in aqueous protein dispersions have been proposed. Further experiments are required to test this hypothesis.

ABBREVIATIONS USED

SMP, spray-dried skimmed milk powder; WPC, whey protein concentrate; CCP, colloidal calcium phosphate; SMUF, synthetic milk ultrafiltrate; EDTA, ethylenediaminetetraacetic acid; ANS, 8-analino-1-naphthalenesulfonic acid; CE, capillary electrophoresis; BLG, β-lactoglobulin; ALA, α-lactalbumin; NEM, *N*-ethylmaleimide.

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